Applicant: Adolfo J. de Bold
Serial No.: 10/712,335
Associate's Reference No.: 08885380US1

Filed: November 13, 2003

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## Amendments to the Specification:

Please insert the paper copy of the Sequence Listing filed herewith following the Drawings.

Please replace the paragraph at page 5, line 10, with the following amended paragraph:

The present invention also embraces the above method (Method A), wherein the BNP is mature BNP or a fragment thereof, ProBNP or a fragment thereof, or a combination of mature BNP and ProBNP, and where ProBNP comprises BNP<sub>1-76</sub>, BNP<sub>1-25</sub>, or BNP<sub>52-76</sub> (SEQ ID NO:1), and mature BNP comprises BNP<sub>77-108</sub>, or a combination of two or more of BNP<sub>1-76</sub>, BNP<sub>1-25</sub>, or BNP<sub>52-76</sub> (SEQ ID NO:1), and BNP<sub>77-108</sub>. Preferably the BNP is BNP<sub>77-108</sub>.

Please replace the paragraph beginning at page 9, line 20 with the following amended paragraph:

As used herein, ProBNP refers to the entire 1-108 amino acid sequence of the brain natriuretic peptide, and it is also referred to as BNP<sub>1-108</sub>. Specific fragments of BNP are referred to based on the numbering of the ProBNP molecule. For example, the mature, 32 amino acid, active BNP portion is referred to as BNP<sub>77-108</sub>, other fragments used within this invention include the N-terminal portion of ProBNP, for example, which is not to be considered limiting in any manner, BNP<sub>1-25</sub>, and the C-terminal region of the cleaved portion of BNP, for example but not limited to, BNP<sub>52-76</sub> (SEQ ID NO:1), derived from the N-terminal region of ProBNP. However, other portions of the ProBNP or BNP molecule may also be suitable for the method of this invention and may be readily determined by one of skill in the art.

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Please replace the paragraph beginning on page 15, line 25 with the following amended paragraph:

Peptides to develop antibodies and to construct standard curves for the radioimmunoassay procedure were custom synthesized commercially. Antibodies were developed against ANF<sub>1-25</sub>, ANF<sub>74-98</sub>, ANF<sub>99-126</sub>, BNP<sub>1-25</sub>, BNP<sub>52-76</sub> (SEQ ID NO:1), and BNP<sub>77</sub>. Peptides regions used to construct standard curves and develop antibodies are depicted in Figure 3 with sequence numbering derived from the peptide prosequences.

Please replace the paragraph beginning on page 15, line 33 (which carries over to page 16, line 19), with the following amended paragraph:

Synthetic ANF<sub>1-25</sub>, ANF<sub>74-98</sub>, ANF<sub>99-126</sub>, BNP<sub>1-25</sub>, BNP<sub>52-76</sub> (SEQ ID NO:1), and BNP<sub>77-108</sub> are produced and each are separately coupled to bovine thyroglobulin using the carbodiimide method (Skowsky WR et al. 1972, J Lab Clin Med 80:134-144, which is incorported incorporated herein by reference) to form corresponding antigen-thyroglobulin conjugates. Female BALBoC mice are separately immunized with each antigen-thyroglobulin conjugate mixed with 100 µg of murarnyl peptide, dissolved in phosphate buffered saline. Protocols for immunization and for the fusion of spleen cells from the immunized mice with cells of the nonsecreting mouse SP2-O plasmacytoma line are described in detail, elsewhere (Milne RW. et al 1992, in Immunological Methods for Studying and Quantifying Lipoproteins and Apolipoproteins, Converse CA and Skinner ER eds, Oxford U Press, pp 61-84, which is incorporated herein by reference). Two methods of screening are used to identify specific antibodies in the culture supernatants of hybridomas. In the first protocol, an antigen, for example any one of ANF<sub>1-25</sub>, ANF<sub>74-98</sub>, ANF<sub>99-126</sub>, BNP<sub>1-25</sub>, BNP<sub>52-76</sub> (SEQ ID NO:1), and BNP<sub>77</sub>. 108, is adsorbed to polystyrene Removal Wells (Dynatech) and after washing and saturation of the wells, they are successively exposed to hybridoma culture supernatant and 1251-anti-mouse IgG. In the second protocol, wells are coated with affinity-purified, anti-mouse IgG and then successively exposed to hybridoma culture supernatant and to a radiolabelled antigen, for example, but not limited to, 125 I-BNP 52-76 (SEQ ID NO:1), as appropriate. Details of the two

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screening protocols have been reported along with a discussion of their relative merits (Milne RW. et al 1992, in Immunological Methods for Studying and Quantifying Lipoproteins and Apolipoproteins, Converse CA and Skinner ER eds, Oxford U Press, pp 61-84, which is incorporated herein by reference).

Please replace the paragraph beginning at page 17, line 30 with the following amended paragraph:

Two 10 mL blood samples are collected per patient. The blood is centrifuged immediately at 4°C and the plasma kept at -80°C. One sample is extracted as described below, for determination of ANF<sub>99-126</sub> and BNP<sub>77-108</sub>. The plasma of the second tube is used for analysis using assays for proBNP fragments (BNP<sub>1-25</sub> and BNP<sub>52-76</sub> (SEQ ID NO:1)) and proANF fragments (ANF<sub>1-25</sub> and ANF<sub>74-98</sub>).

Please replace the paragraph beginning at page 18, line 21 with the following amended paragraph:

For the purposes of developing a simple method to measure NT-BNP, an ELISA protocol is pursued. Two approaches are possible for measuring soluble antigens with ELISAs: 1. Technology based on a direct competitive protocol, or, 2. Technology based on a "sandwich" technique involving a competitive reaction plus a reaction with a second antibody directed to a second epitope in the antigen. A capture and secondary polyclonal and monoclonal antibodies against the N-terminal portion of BNP<sub>52-76</sub> used is:

Lys-Ser-Arg-Glu-Val-Ala-Thr-Glu-Gly-lle-Arg-Gly-His-His-Arg-Lys-Met-Val-Leu-Tyr-Thr-Leu-Arg-Ala-Pro-Arg (SEQ ID NO:1)

The BNP<sub>77-108</sub> fragment may also be used (Figure 1)